Coding of odors in the anterior olfactory nucleus

Coding of odours in the anterior olfactory nucleus.

Takahiro Tsuji[1], Chiharu Tsuji[1], Maja Lozic[1], Mike Ludwig[1,2] and Gareth Leng[1]

[1]Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, UK

[2]Centre for Neuroendocrinology, University of Pretoria, Pretoria, South Africa

***Corresponding author:** Mike Ludwig, Centre for Discovery Brain Sciences, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK

Email: mike.ludwig@ed.ac.uk

Keywords: anterior olfactory nucleus, hazard function, interspike interval, oscillatory firing, vasopressin

Abstract

Odorant molecules stimulate olfactory receptor neurones, and axons of these neurones project into the main olfactory bulb where they synapse onto mitral and tufted cells. These project to the primary olfactory cortex including the anterior olfactory nucleus (AON), the piriform cortex, amygdala and the entorhinal cortex. The properties of mitral cells have been investigated extensively, but how odour information is processed in subsequent brain regions is less well known. In the present study, we recorded the electrical activity

of AON neurones in anesthetized rats. Most AON cells fired in bursts of 2-10 spikes separated by very short intervals (<20 ms), in a period linked to the respiratory rhythm. Simultaneous recordings from adjacent neurones revealed that the rhythms of adjacent cells, while locked to the same underlying rhythm, showed marked differences in phase. We studied the responses of AON cells to brief high frequency stimulation of the LOT, mimicking brief activation of mitral cells by odour. In different cells, such bursts evoked transient or sustained bursts during stimulation or, more commonly, post-stimulation bursts after inhibition during stimulation. This suggests that, in AON cells, phase shifts occur as a result of post-inhibitory rebound firing, following inhibition by mitral cell input, and we discuss how this supports processing of odour information in the olfactory pathway. Cells were tested for their responsiveness to a social odour (the bedding of a strange male) amongst other simple and complex odours tested. Eleven cells responded strongly and repeatable to bedding odour, and these responses were diverse, including excitation (transient or sustained), inhibition, and activation after odour presentation, indicating that AON neurones respond not only to the type of complex odour but also to temporal features of odour application.

Abbreviations: AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; MOB, main olfactory bulb; IoD, index of dispersion; ISI, interspike interval; LOT, lateral olfactory tract;

Competing Interests Statement: The authors declare that there are no conflicts of interest.

Funding information: Supported by grants from the Biotechnology and Biological Research Council (BB/J004723/1) and Medical Research Council (MR/M022838/1) (ML, GL), and fellowships from the Japanese Society for the Promotion of Science (TT, CT).

Introduction

Olfactory information is transduced when odorant molecules contact the dendrites of olfactory receptor neurones in the olfactory epithelium of the dorsal nasal cavity (Buck & Axel, 1991). These neurones project to the glomerular layer of the main olfactory bulb (MOB) (Mombaerts *et al.*, 1996) where they synapse onto the dendrites of mitral and tufted cells, the output neurones of the olfactory bulb (Shipley & Ennis, 1996; Menini *et al.*, 2004; Lledo *et al.*, 2005). Each mitral cell sends its apical dendrite to a single glomerulus, and this receives inputs from a single class of olfactory receptor neurones (Bargmann, 2006); the mitral cells are thus tuned to respond to odorants that activate a single type of odorant receptor. However, the olfactory sensory neurones are also mechanosensitive (Grosmaitre *et al.*, 2007), and are activated rhythmically by respiratory activity (Duchamp-Viret *et al.*, 2005). Accordingly, the spontaneous spiking activity of many mitral cells follows the respiratory cycle, with peak firing rates occurring typically late in the inspiratory phase in rats (Buonviso *et al.*, 2003; Margrie & Schaefer, 2003). In the presence of an odour that activates (or inhibits) the mitral cell, this cyclic activity is amplified (or attenuated). Thus for each mitral cell, odour information is coded as the amplitude (relative to a basal amplitude) of a signal that oscillates with the respiratory rhythm.

By contrast to mitral cells, tufted cells are more excitable, and respond with a shorter latency to stimulation of olfactory sensory neurones (Griff *et al.*, 2008; Burton & Urban, 2014; Geramita & Urban, 2017; Vaaga & Westbrook, 2017), hence those that show a clear respiratory rhythm are active earlier in the respiratory cycle than mitral cells, and whereas mitral cells show sustained responses to sensory stimulation, the responses of tufted cells adapt.

Axons from the mitral and tufted cells converge to form the lateral olfactory tract (LOT), and collaterals of these axons innervate the anterior olfactory nucleus (AON) an important second-order processing station in the olfactory pathway (Brunjes *et al.*, 2005; Sosulski *et al.*, 2011). Individual cells in the AON receive inputs from mitral cells whose dendrites arise in different glomeruli, and hence they are more broadly tuned than mitral or tufted cells. Transynaptic labelling studies in mice suggest that individual AON cells receive inputs from at least four mitral cells (Miyamichi *et al.*, 2011). In mice, it appears that mitral and tufted cells may project to different regions of the AON (Igarashi *et al.*, 2012).

In urethane-anesthetised rats, mitral cells show complex patterns of spontaneous activity: the mean firing rate is about 6.4 spikes/s, and comprises long bursts (of ~154s) at ~11 spikes/s separated by long silent periods (~ 100s) that are not synchronized amongst even neighbouring mitral cells (Leng *et al.*, 2014). Within bursts, activity in most mitral cells oscillates with a period of 400-600ms, reflecting peak activation during the inspiration phase of respiration, and excitatory responses to odours are typically apparent as an intensification of this respiratory-locked oscillatory discharge. The significance of the long bursting appears to be that cells that alternate between activity and silence can optimally detect both excitatory stimuli (in the silent phase) and inhibitory stimuli (in the active phase).

The AON is a very heterogeneous nucleus. It contains pyramidal glutamatergic cells and a variety of interneurons, many of which are GABA-ergic, but which also contain a considerable diversity of neuropeptides (Kay & Brunjes, 2014). One role of the AON is to assemble the inputs of activated glomeruli into a representation of complex odorants, and one particular role for the AON may be in processing socially relevant

101 complex odours. Whereas the output cells of the MOB respond selectively to a narrow
102 range of odours, in mice most pyramidal AON neurones can be activated by mixtures of
103 structurally dissimilar components (Lei *et al.*, 2006). The response of an AON neurone
104 to an effective mixture often exceeds the sum of its responses to the components,
105 indicating a nonlinear combinatorial interaction.

106 Here, we used *in vivo* electrophysiology to study how inputs from the olfactory
107 bulb are processed in the AON. We studied the spontaneous discharge patterning of AON
108 neurones to identify subpopulations and studied their responsiveness to stimulation of the
109 LOT. To investigate neuronal responses to a social odour, we impregnated air with the
110 odour from the soiled bedding of the cage of a novel adult male rat and applied this odour
111 in an air stream to the nose as well as testing other simple and complex odours.

112

113 **Material and Methods**

114 ***Ethical Approval***

115 Procedures conducted in the UK were approved by the local Ethics Committee
116 and the UK Home Office under the Animals Scientific Procedures Act 1986.

117

118 ***Electrophysiology***

119 Single neurones were recorded from 120 adult male Sprague-Dawley rats (250-
120 400 g) anesthetized with urethane (ethyl carbamate, 1.3 g/kg i.p.) using conventional
121 extracellular recording techniques. In some experiments, respiration was monitored using
122 a Pulse Transducer (TN1012/ST, Adinstruments, Oxford, UK). A recording electrode
123 (glass micropipette filled with 0.9% NaCl, 20-40 MΩ) was lowered into the dorsally
124 exposed AON, 1.0-2.5 mm lateral to the midline and 3.8-4.7 mm anterior to bregma. We

125 recorded neural activity through an amplifier (Axopatch 200b, Molecular Device,
126 Sunnyvale, USA) and processed them by Spike2 software (Cambridge Electronic Design
127 Limited, Cambridge, UK). In initial experiments, the position of the recording electrode
128 was verified by visualizing incorporated neurobiotin in the recorded cell by juxtacellular
129 labelling (Sabatier & Leng, 2008) (Fig. 1A,B). Labelled cells were consistently found in
130 the main, ventrolateral portion of the AON, and were multipolar with oval cell bodies.
131 To stimulate the LOT, a concentric bipolar stimulating electrode (SNEX-100,
132 Clark Electromedical Instrument, Kent, UK) was positioned on the ipsilateral LOT, via a
133 burr hole in the dorsal surface of the skull, 1.4mm posterior to bregma, 3.2mm lateral to
134 midline, 9.5mm deep (Paxinous & Watson, 2006). Electrical stimulation (1-ms matched
135 biphasic pulses, 1mA peak-to-peak) was generated by a GRASS S88 stimulator with
136 stimulus isolation and constant current units (Grass Products, Warwick, USA) as
137 previously (Leng *et al.*, 2014). In initial experiments the position of the stimulating
138 electrode was confirmed histologically and by verifying that stimulation at this site
139 evoked the expected potential changes in the mitral cell layer of the main olfactory bulb
140 (Leng *et al.,* 2014).
141 In some cases, double recordings were made with the single recording electrode.
142 When the spike heights were sufficiently different, these were sorted using the LabSpike
143 software generated by (Bhumbra *et al.*, 2004) and available from the CED website
144 (http://www.ced.co.uk/upu.shtml).
145 Odours were applied through a polythene cannula (0.1mm diameter) placed
146 3mm in front of the nose of the rat. Beddings were from male rat cages different from the
147 cages of the experimental rats. For each test, 20ml of odour-saturated air was applied to
148 the nose over ~5s using a 50-ml syringe. Some cells were tested with a range of odours,

including heptanal, hexanal, valderaldehyde, lemon, garlic and peppermint, and when responses were observed, the specificity was checked by a similar application of odour-free air. For cells that showed a response to bedding odour, activity in 0.5-s bins was compared 20s before and in the first 10s after bedding was applied.

Statistical analysis

Autocorrelation histograms, ISI distributions and peri-stimulus time histograms were constructed in Spike2. Autocorrelation histograms were constructed from 300s of spontaneous activity in 100-ms bins, and normalised autocorrelation histograms were normalised to the total number of spikes. ISI histograms were constructed in 10-ms bins from at least 300s of stable spontaneous discharge activity. To generate population averages (consensus ISI distributions), each ISI distribution was normalised to the total number of ISIs. We measured index of dispersion (IoD) of firing rates in 10-s bins over 300s of spontaneous activity for each cell as the ratio of the variance to the mean.

We used two ways to assess the relationship of spiking activity to respiratory rhythm. First, we looked at spike activity relative to the respiratory cycle, using the wavemark function in Spike2 to mark each respiratory cycle at a consistent stage, and using this as a trigger, constructed a histogram of spike activity relative to this trigger and used the same trigger to plot the average respiratory waveform. Second, we looked at the respiratory waveform relative to spike activity, using each recorded spike as a trigger for the waveform average: this spike-triggered waveform is thus constructed in a way strictly analogous to the construction of the autocorrelation histogram.

Results

Our first objective was to describe the spontaneous firing patterns of AON neurones and test them for responsiveness to bedding odour and for their responsiveness to LOT stimulation. We initially analysed 240 spontaneously active AON cells recorded from 103 rats. Their firing rates ranged from 0.1 to 32.4 spikes s^{-1}, with a mean (SEM) of 5.8 ± 0.37 spikes s^{-1}. Although the spontaneous firing rates were thus generally low, instantaneous frequencies in most cells occasionally exceeded 100 spikes s^{-1} (Fig. 1C) indicating that the low spontaneous rates did not reflect an inability to discharge at high frequencies. Most cells fired in intermittent short bursts (Fig. 1D) that often were associated with rhythmic changes in the background field potential (Fig. 1E). The firing rates of AON cells were distributed unimodally, and subpopulations could not be distinguished from this alone (Fig. 1F). For AON cells generally, the standard deviation of ISIs was proportional to the mean firing rate, revealing no subpopulations (Fig. 1G).

Rhythmic and arrhythmic cells

For each cell, we constructed autocorrelation histograms over 300s or more of stable spontaneous activity (Fig. 2B). This allowed us to identify three large subpopulations. The largest of these, 147 *type 1 rhythmic cells* (Fig. 2B), fired at 6.8 ± 0.5 spikes s^{-1} (range 0.18-25.1) and showed cyclic activity characterised by regular peaks in the autocorrelation histogram separated by intervals of a constant duration (mean 0.61 ± 0.008 s; Fig. 2C). This is similar to the periodicity of rhythmic oscillations in mitral cells and corresponds to the mean respiratory rate of urethane-anesthetized rats (85-110 min^{-1}). Activity mainly comprised bursts of 2-10 spikes separated by ISIs of < 25 ms in each phase of the cycle. About half (70) of the type 1 cells showed virtually no activity between bursts, while the remainder showed sparse spikes between bursts. The ISI

distributions of type 1 cells were all similarly skewed and were unimodal in the range 0-200 ms with a mean mode of 17.1 ± 0.9 ms (median 16), (Fig. 2D,F). Four cells discharged with periods of 300-400 ms, approximately half that of the mean respiratory rhythm (not shown). In some recordings, spikes from two neurones could be readily separated by spike height and waveform. In all cases (10 pairs), the two cells were locked to the same underlying rhythm, but their discharge activity could be either in phase or out of phase with each other (Fig. 2E).

A second subpopulation, 45 *type 2 rhythmic cells* (Fig. 3) fired at 3.3 ± 06 spikes/s (range 0.1-21.6 spikes/s) and showed cyclic activity with a mean period approximately twice that of type 1 cells. Again, activity mainly comprised bursts of 2-10 spikes separated by ISIs of < 25ms, with sparse spiking between bursts (Fig. 3A). In the typical type 2 cell shown in Figure 3A, we analysed 911 bursts from 1352s of spontaneous activity, defining bursts by the first ISI < 300ms after an ISI of > 500ms. Bursts contained 2-25 spikes (mean (SD) 8(5.1) spikes; burst length 451 (287) ms; period 1.47 (0.46) s; Fig. 3B). There was no frequency adaptation within bursts: the mean (SD) interval between the first two spikes in bursts was 49 (48) ms (n=911) and that between the 15th and 16th spikes was 47 (44) ms (n=96). The ISI distribution was unimodal with a mode of 23ms (Fig. 3C), and the autocorrelation (Fig. 3D) showed peaks at intervals of 1.2 s. The normalized average autocorrelation for all type 2 cells (Fig. 3E), constructed as for type 1 cells, showed no repeated peaks as the periods varied too much between cells. Like type 1 cells, all type 2 cells had ISI distributions that were unimodal in the range 0-300 ms, with a mean mode of 17.2 ± 2.2 ms (median 13.5 ms; Fig. 3F).

A third population of 35 *arrhythmic cells* fired at 6.0 ± 1.4 spikes/s (range 0.1-32.4) with no apparent oscillatory activity. The ISI distributions again were unimodal in

221 the range 0-300ms, with a mean mode of 17.5 ± 2.2 ms, but activity in these cells was
222 irregular, with no clear bursting pattern. This group includes slow firing cells where a
223 rhythm may not have been apparent because of the low discharge rate.
224
225 ***Isoperiodic bursting***
226 The discharge rate of many AON cells displayed intermittent long bursts
227 superimposed on a sustained background activity. To quantify this, we measured the index
228 of dispersion (IoD) of firing rates in 10-s bins over 300s of spontaneous activity for each
229 cell. For a cell firing randomly at a constant mean rate, the IoD should equal one
230 regardless of firing rate: of the 248 cells, only 82 had an IoD below 2, while for 80 the
231 IoD exceeded 7.
232 Some cells displayed repeated, long bursts from which we could measure burst
233 and interburst durations. We analysed 20 such cells (19 type 1 cells and 1 type 2 cell) with
234 a mean IoD of 29.5 ± 4.9). The mean intraburst firing rate was 7.7 ± 1.0 spikes/s (median
235 6.7, range 1.3-19.8) for bursts of 111 ± 11s (median 92s, range 50-220s). The interburst
236 firing rates were 1.6 ± 0.4 spikes/s (median 0.9, range 0.2-7.5) for intervals of 110 ± 22 s
237 (median 93s, range 43-218s). These are close to the burst and interburst durations of
238 mitral cells in the MOB (bursts, 122 ± 10 s; interburst intervals, 129 ± 11 s), but intraburst
239 firing rates were lower than the mean of 14.3 spikes/s in mitral cells (Leng *et al.*, 2014).
240
241 ***Responses to social odour***
242 We applied the smell of bedding from conspecific male rats to the anesthetized
243 male rats. Of 179 AON cells tested, 14 cells (all type 1) responded strongly and repeatedly
244 to this but not to similar application of clean air (Fig. 4); nine cells were activated (Fig.

4A,B) and four were inhibited (Fig. 4C). In the nine excited cells (Fig. 4B), the mean firing rate increased from 3.9 ± 1.2 spikes s^{-1} (in the10 s before the application) to 11.2 ± 2.6 spikes.s^{-1} (for 10 s during bedding application) and went back to 5.5 ± 1.8 spikes.s^{-1} (for 10 s after application). One cell unaffected during odour application was strongly activated after the end of odour application (Fig. 4D), and two inhibited cells showed a strong excitatory rebound after the end of odour application (Fig. 4E). Similar complex responses to either the presentation of an odour or to its removal were seen in other cells in response to odours other than bedding (not shown).

Seven cells activated by bedding odour responded to one or more of the other odours tested (five cells were tested with valderaldehyde of which four responded, four cells were tested with hexanol of which two responded strongly and one weakly).

Responses to electrical stimulation of the LOT

Of 26 type 1 cells, ten responded to 1-Hz stimulation of the LOT with clear orthodromic excitation at latencies between 7 and 20 ms (Fig. 5A,B). The other 16 were inhibited, with apparently similar latencies, although determining latency accurately in inhibited cells was generally imprecise.

In response to brief high-frequency stimulation, ten type 1 cells tested displayed virtually complete inhibition during stimulation (Fig. 5C). In six cells, activity swiftly returned to normal after the end of stimulation, and in four there was continued inhibition for several seconds (Fig. 5E). In another 12 cells, stimulation evoked a strong excitatory response. In six cases, this arose as a post-stimulation excitation after inhibition throughout the stimulation (Fig. 5D), in two cases, activation throughout stimulation was followed by a strong inhibition (Fig. 5E,F,H,I). In two cases, activation began during the

stimulation after initial inhibition, and outlasted the stimulation by several seconds (Fig. 5G), and in two cases there was persistent activation in neurones excited throughout stimulation.

Thus all type 1 neurones tested responded strongly to LOT stimulation, in most cases with a short latency, but had mixed inhibitory and excitatory effects, including effects that outlasted stimulation by several seconds.

Figure 6 compares the response of one type 1 cell to bedding with its response to LOT stimulation. This cell was repeatably and strongly activated by bedding odour, weakly activated by garlic, and unresponsive to air or vanilla extract (Fig. 6A). The response to bedding comprised intensified cyclic bursting (Fig. 6B). The same cell was tested for its response to 500 ms of LOT stimulation at between 20 and 200 Hz; it responded to stimulation at 150 and 200 Hz with a strong after-discharge, but showed no response to lower frequencies (Fig. 6C). We then tested it in response to prolonged stimulation at 5, 10 and 20 Hz (Fig. 6D). Stimulation at 10 or 20 Hz was initially ineffective, but progressively revealed a short latency excitation (Fig. 6E,F). This is consistent with a progressive enhancement of synaptic excitation, or with the superposition of constant excitation and a progressively waning synaptic inhibition.

All type 2 cells tested also responded to LOT stimulation, and again the responses varied considerably. The most common responses were inhibitions followed by excitation, and the effects varied over time during continued stimulation (Fig. 7). Of 13 type 2 cells tested with brief high-frequency stimulation (50 Hz, 0.5 s), six showed strong post-stimulus excitation and seven showed strong post-stimulus inhibition.

Comparison with the respiratory cycle.

293 Thus these initial experiments showed that most of the AON neurones that we
294 encountered showed strong cyclic activity presumed to reflect inputs locked to respiration,
295 but it was clear from the occasional double recordings that different neurones fired with
296 different phase relationships. It was also clear from the studies with LOT stimulation that
297 some AON neurones showed short latency excitation, consistent with a direct excitatory
298 input from the LOT. However, more neurones were inhibited by LOT stimulation, and
299 many showed complex responses to trains of stimulation. This suggesting that their
300 responses were mainly mediated indirectly, presumably involving GABA inputs from
301 neurones within the LOT that were themselves activated by LOT stimulation.
302 In subsequent experiments we therefore focussed on recording additional type 1
303 cells, characterised the relationship of their cyclic activity to the respiratory rhythm, and
304 compared this with their responsiveness to LOT stimulation.
305 We recorded from a further 28 type 1 cells in 17 rats. For each of these we
306 constructed autocorrelation histograms, ISI distributions, and we used two ways to assess
307 the relationship of spiking activity to respiratory rhythm. Nine cells showed cyclic
308 activity beginning in the inspiratory phase of respiration, and nineteen cells showed cyclic
309 activity beginning in the expiratory phase. We could classify 20 of these neurones by their
310 short-latency responses to LOT stimulation; five as being excited by LOT stimulation and
311 15 as being inhibited. All of the five excited neurones were inspiration neurones (Fig. 8);
312 13 of the 15 inhibited neurones were expiration neurones (Fig. 9).
313
314 **Discussion**
315 Processed odour information is transmitted from the MOB via the LOT to the
316 AON (Shipley & Ennis, 1996; Menini *et al.*, 2004; Lledo *et al.*, 2005). The AON

317 distributes the information to the contralateral olfactory bulb and piriform cortex and
318 engages in reciprocal interactions with the ipsilateral bulb and cortex (Mori *et al.*, 1979;
319 Kikuta *et al.*, 2010; Miyamichi *et al.*, 2011; Sosulski *et al.*, 2011).

320 From the present results, we can draw some inferences about how the AON
321 processes the synaptic input from the MOB. In similar experimental conditions to those
322 used here, the mitral cells and tufted cells of the olfactory bulb display rhythmic spiking
323 activity locked to the phase of respiration. This input to the AON is purely excitatory, as
324 mitral cells and tufted cells are all glutamatergic. Many AON cells are GABAergic, with
325 intrinsic projections within the AON (Kay & Brunjes, 2014). Accordingly, cells in the
326 AON are expected to be excited by LOT stimulation if they receive a direct input from
327 the MOB (primary AON neurones), but to be inhibited if they receive inhibitory inputs
328 from primary recipients. Of course it is to be expected that many AON neurones will
329 receive both direct excitatory inputs and indirect inhibitory inputs.

330 The first conspicuous feature of these results is that a high proportion of AON
331 neurones display spontaneous cyclic activity tightly locked to the respiratory rhythm. This
332 locking is apparent in the clarity of cycles displayed by the autocorrelation histograms –
333 indeed the cyclicity of AON neurones is more marked than that of mitral cells recorded
334 in similar experimental conditions.

335 Mitral cells in the anesthetized rat alternate between long periods of activity and
336 silence. During active phases, their mean discharge rate is 11 spikes/s, and, in cells in
337 which a respiratory rhythm is apparent, this typically oscillates between 5 and 20 spikes/s
338 over the respiratory cycle. AON cells are much quieter but display bursts of spikes locked
339 to the respiratory cycle. The bursts generally comprise just a few spikes, but as these
340 bursts are separated by long silent intervals, the periodic activity is much more marked

than that of mitral cells, which are generally active throughout the respiratory rhythm. The rhythms of type 1 cells were thus more clearly defined than those of mitral cells (Leng *et al.*, 2014) because of the sparsity of spikes between bursts. In effect, the respiratory rhythms of AON cells are more marked because the AON operates as a high-pass filter of the MOB input.

As the respiratory rhythm must reach the AON via the output cells of the olfactory bulb, we conclude that the spontaneous cyclic activity of these AON cells is primarily driven by direct or indirect inputs from output cells of the olfactory bulb whose activity is locked to the same phase of the respiratory cycle. As stated in the introduction, there is evidence that mitral cells in the rat are generally activated late in the inspiration cycle, but not all studies have reported this; for example, Phillips *et al.*, reported that mitral and tufted cells are activated at diverse phases of an artificial respiratory cycle (Phillips *et al.*, 2012). Thus we must consider the possibility that AON neurones that are active during inspiration receive inputs from MOB neurones that are activated during inspiration, while AON neurones active during expiration receive inputs from MOB cells that are activate during expiration. However, if so we would expect to find that AON neurones were activated by LOT stimulation regardless of how their activity was linked to respiration. Instead, we found that AON neurones that were active during inspiration were excited by LOT stimulation, whereas most of those active during expiration were inhibited by LOT stimulation.

Mitral cells typically respond to odours with either inhibition or excitation that is sustained throughout odour application, and in excited cells that display a respiratory rhythm the response involves an intensification of that rhythm (Leng et al. 2014). Mitral cells are also narrowly tuned, responding only to closely-related odours. Here we used a

365 complex odour – the odour of male rat bedding. We chose this odour as a naturalistic
366 odour of considerable social relevance to rats, of a type that the AON is thought to be
367 particularly important for processing. We had previously tested this odour in our studies
368 of mitral cells but without finding any responsive cells, possibly because we simply were
369 not recording in responsive regions of the bulb (Leng *et al.*, 2014): in mice, the existence
370 of a "specialist glomerulus" narrowly tuned to a compound present in urine has been
371 reported (Lin et al., 2005). In the AON, we found quite a substantial number of cells
372 responsive to bedding odour. The responses were generally complex, including transient
373 "on" responses and marked "off" responses, and responsive cells were typically similarly
374 responsive to valderaldehyde and/or hexanol – the only two 'simple' odours that we tested.
375 A recent fMRI study in rats (Zhao *et al.*, 2017) found no olfactory adaptation to
376 prolonged (200-s) odour application in either the olfactory bulb or the AON, but found
377 strong adaptation in the piriform cortex, and transient responses have recently been
378 reported to be common for odour-responsive neurones in the mouse piriform cortex
379 (Tantirigama *et al.*, 2017). In the AON, we observed complex non-linearities, varying
380 between AON cells, in their response to mitral cell input, including both frequency-
381 dependent non-linearities, which may amplify odour responses, and temporal non-
382 linearities arising presumably from slow activity-dependent effects which in some cells
383 produce "on" and "off" responses to odour stimuli in some cells.
384 We tested AON cells with brief stimulation of the LOT, which antidromically
385 activates mitral cells and their collaterals to the AON. In some of these experiments we
386 stimulated at 0.5 s for 50 Hz, to mimic the activation during an inhibitory phase of the
387 respiratory cycle as observed during odour application. Such stimulation commonly
388 produced either a burst of activity during stimulation, or an inhibition during stimulation

followed by a burst after stimulation. The latter effect suggests an obvious explanation of how AON cells display rhythmic firing at different phases of the respiration cycle – as a rebound excitation following inhibition.

A feature of the AON was the many cells (the type 2 cells) showed periodic activity with a period apparently twice that of the respiratory rhythm – confirmed in examples recorded in conjunction with recordings of respiratory activity (not shown). This seems likely to occur as a result of prolonged post-activation inhibition that occludes the excitatory input on alternate cycles. If so, the post-activation inhibition is not plausibly accounted for by spike-dependent afterhyperpolarisation intrinsic to type 2 cells, which typically discharged only a few spikes in each burst, but probably arises from aggregate inhibition by quasi-synchronously activated interneurons.

Thus AON neurones represent the rhythmic input from the mitral cells as periodic activation with a varied phase relationship to the original input, depending on the balance between excitation from the mitral cell input, inhibition from intrinsic neurones, and rebound excitation following inhibition, and this is reflected in the diverse phase relationships of periodic activity in adjacent mitral cells.

The spontaneous activity of mitral cells generally oscillates with peaks of activity in the inspiration phase of the respiratory cycle, and in the presence of an odour, MOB cells responsive to that odour discharge more intensely during inspiration. Neurones in the AON however receive inputs derived, directly or indirectly, from many mitral/tufted cells, only a few of which will be activated by a given odour. Considering the aggregate input, the response of the odour responsive cells will be ‘diluted’ by the synchronous, spontaneous discharge of the non-responsive MOB cells. Such a population is simulated in Figure 10. In this simulation, one of eleven cells is activated strongly by

413 an ’odour’ to increase its activity three fold during bursts, but in the aggregate activity of
414 the population this is imperceptible. The aggregate burst activation increases by less than
415 10%, within the range of random variation, and it is hard to see how any processing of
416 this aggregate signal could reliably extract the odour signal. However, it appears that
417 odour processing in the AON involves ‘phase shifting’ the MOB input by variable
418 amounts, in addition to a non-linearity of response that yields a pattern of short bursts,
419 linked to the respiratory cycle, with no activity between bursts. If we look at the aggregate
420 activity of such a population (Figure 10), the response to odour stimulation of one of the
421 11 cells is clearly apparent as a 50% increase above a stable background rate. Not only is
422 the signal: noise ratio protected, but the pattern of response is now readily amenable to
423 signal extraction by neurones that response non-linearly to this input.

424 In summary, the processing by AON neurones of the input from the MOB
425 appears to include four distinct operations. First, there is convergence of inputs, so that
426 AON cells are broadly tuned. Second, processing acts as a high-pass filter: AON neurones
427 have a low spontaneous firing rate but because they respond non-linearly to the MOB
428 input, they retain a marked rhythm in the form of short bursts with a period matching the
429 respiratory rhythm. Third, this rhythm is phase shifted in many cells, apparently by a
430 combination of inhibition and post-inhibitory rebound activation. This phase shifting
431 seems likely to help maintain the signal to noise ratio of odour responses in the population
432 output.

433

434 **Author contributions:** C.T., T.T, Ma.L., M.L. and G.L. designed the research; C.T.,
435 T.T. and Ma.L. performed the research; T.T. and G.L. analysed data; and G.L., T.T., C.T.
436 and M.L. wrote the paper.

437

438 **References**

439 Bargmann CI. (2006). Comparative chemosensation from receptors to ecology. *Nature*
440 **444,** 295-301.

441

442 Bhumbra GS, Inyushkin AN & Dyball RE. (2004). Assessment of spike activity in the
443 supraoptic nucleus. *J Neuroendocrinol* **16,** 390-397.

444

445 Brunjes PC, Illig KR & Meyer EA. (2005). A field guide to the anterior olfactory nucleus
446 (cortex). *Brain Res Rev* **50,** 305-335.

447

448 Buck L & Axel R. (1991). A novel multigene family may encode odorant receptors: a
449 molecular basis for odor recognition. *Cell* **65,** 175-187.

450

451 Buonviso N, Amat C, Litaudon P, Roux S, Royet JP, Farget V & Sicard G. (2003). Rhythm
452 sequence through the olfactory bulb layers during the time window of a
453 respiratory cycle. *Eur J Neurosci* **17,** 1811-1819.

454

455 Burton SD & Urban NN. (2014). Greater excitability and firing irregularity of tufted cells
456 underlies distinct afferent-evoked activity of olfactory bulb mitral and tufted cells.
457 *J Physiol* **592,** 2097-2118.

458

459 Duchamp-Viret P, Kostal L, Chaput M, Lansky P & Rospars JP. (2005). Patterns of
460 spontaneous activity in single rat olfactory receptor neurons are different in

normally breathing and tracheotomized animals. *J Neurobiol* **65,** 97-114.

Geramita M & Urban NN. (2017). Differences in glomerular-layer-mediated feedforward inhibition onto mitral and tufted cells lead to distinct modes of intensity coding. *J Neurosci* **37,** 1428-1438.

Griff ER, Mafhouz M & Chaput MA. (2008). Comparison of identified mitral and tufted cells in freely breathing rats: II. Odor-evoked responses. *Chem Senses* **33,** 793-802.

Grosmaitre X, Santarelli LC, Tan J, Luo M & Ma M. (2007). Dual functions of mammalian olfactory sensory neurons as odor detectors and mechanical sensors. *Nat Neurosci* **10,** 348-354.

Igarashi KM, Ieki N, An M, Yamaguchi Y, Nagayama S, Kobayakawa K, Kobayakawa R, Tanifuji M, Sakano H, Chen WR & Mori K. (2012). Parallel mitral and tufted cell pathways route distinct odor information to different targets in the olfactory cortex. *J Neurosci* **32,** 7970-7985.

Kay RB & Brunjes PC. (2014). Diversity among principal and GABAergic neurons of the anterior olfactory nucleus. *Front Cell Neurosci* **8,** 111.

Kikuta S, Sato K, Kashiwadani H, Tsunoda K, Yamasoba T & Mori K. (2010). From the Cover: Neurons in the anterior olfactory nucleus pars externa detect right or left

485 localization of odor sources. *Proc Nat Acad Sci USA* **107,** 12363-12368.
486
487 Lei H, Mooney R & Katz LC. (2006). Synaptic integration of olfactory information in
488 mouse anterior olfactory nucleus. *J Neurosci* **26,** 12023-12032.
489
490 Leng G, Hashimoto H, Tsuji C, Sabatier N & Ludwig M. (2014). Discharge patterning in
491 rat olfactory bulb mitral cells in vivo. *Physiol Rep* **2**, e12021.
492
493 Lin DY, Zhang SZ, Block E & KatzLC. (2005). Encoding social signals in the mouse
494 main olfactory bulb. *Nature* **434,** 470-477.
495
496 Lledo PM, Gheusi G & Vincent JD. (2005). Information processing in the mammalian
497 olfactory system. *Physiol Rev* **85,** 281-317.
498
499 Margrie TW & Schaefer AT. (2003). Theta oscillation coupled spike latencies yield
500 computational vigour in a mammalian sensory system. *J Pphysiol* **546,** 363-374.
501
502 Menini A, Lagostena L & Boccaccio A. (2004). Olfaction: from odorant molecules to the
503 olfactory cortex. *News Physiol Sci* **19,** 101-104.
504
505 Miyamichi K, Amat F, Moussavi F, Wang C, Wickersham I, Wall NR, Taniguchi H, Tasic
506 B, Huang ZJ, He Z, Callaway EM, Horowitz MA & Luo L. (2011). Cortical
507 representations of olfactory input by trans-synaptic tracing. *Nature* **472,** 191-196.
508

509 Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J &
510 Axel R. (1996). Visualizing an olfactory sensory map. *Cell* **87,** 675-686.
511
512 Mori K, Satou M & Takagi SF. (1979). Axonal projection of anterior olfactory nuclear
513 neurons to the olfactory bulb bilaterally. *Exp Neurol* **64,** 295-305.
514
515 Paxinos G & Watson C. (2006). The rat brain stereotaxic coordinates. Academic Press,
516 San Diego, CA.
517
518 Phillips ME, Sachdev RN, Willhite DC & Shepherd GM. (2012). Respiration drives
519 network activity and modulates synaptic and circuit processing of lateral
520 inhibition in the olfactory bulb. *J Neurosci* **32,** 85-98.
521
522 Sabatier N & Leng G. (2008). Spontaneous discharge characteristic of neurons in the
523 ventromedial nucleus of the rat hypothalamus in vivo. *Eur J Neurosci* **28,** 693-
524 706.
525
526 Shipley MT & Ennis M. (1996). Functional organization of olfactory system. *J Neurobiol*
527 **30,** 123-176.
528
529 Sosulski DL, Bloom ML, Cutforth T, Axel R & Datta SR. (2011). Distinct representations
530 of olfactory information in different cortical centres. *Nature* **472,** 213-216.
531
532 Tantirigama ML, Huang HH & Bekkers JM. (2017). Spontaneous activity in the piriform

cortex extends the dynamic range of cortical odor coding. *Proc Nat Acad Sci USA***114,** 2407-2412.

Vaaga CE & Westbrook GL. (2017). Distinct temporal filters in mitral cells and external tufted cells of the olfactory bulb. *J Physiol* **595,** 6349-6362.

Zhao F, Wang X, Zariwala HA, Uslaner JM, Houghton AK, Evelhoch JL, Hostetler E, Winkelmann CT & Hines CD. (2017). fMRI study of the role of glutamate NMDA receptor in the olfactory adaptation in rats: Insights into cellular and molecular mechanisms of olfactory adaptation. *Neuroimage* **149,** 348-360.

Figure legends:

Figure 1. Juxtacellularly labelled neurone in the AON.

A, Juxtacellularly labelled neurone (arrowhead) in the rat AON, shown at higher magnification on the lower. AOB; accessory olfactory bulb, MOB; main olfactory bulb, AONd; anterior olfactory nucleus dorsal, AONl; anterior olfactory nucleus lateral. **B,** Higher magnification of the neurone arrowed in A showing multipolar neurone with oval cell body. **C,** Extract of voltage trace showing individual spikes. This neurone showed decreasing spike height in spikes clustered at high frequency. **D,** Voltage record spontaneous activity pattern showing rhythmic discharge of spikes, including short bursts. **E,** Average action potential from this neurone (average of all spikes fired during 300 s) expanded to show rhythmic oscillations of voltage. **F,** Distribution of spontaneous firing rates of a sample of 240 AON neurones. **G,** The SD of ISIs is closely correlated with the

557 mean ISI (R^2=0.88).

558

559 **Figure 2**. **Type 1 neurones in the AON.**

560 **A,** Extract of voltage trace of a typical type 1 neurone. **B,** Autocorrelation of spike activity
561 of the neurone in A showing discharge activity with a period of ~600 ms. **C,** Normalised
562 autocorrelations from type 1 neurones sorted according to period of rhythm (500-600 ms
563 dotted line, n=67; 600-700 ms solid line, n=50). Means ± SE. **D,** Consensus ISI
564 distribution for 143 type 1 cells (means ± SE). **E,** Pair of type 1 neurones, displaying
565 bursts with a period of about 600 ms. Bursts in the smaller cell (one burst circled in dashed
566 line) are out of phase with bursts in the larger cell (circled in dotted line). **F,** Box plots
567 showing mean and interquartile ranges, median (X) and outliers (circles) for spontaneous
568 firing rates (left and modal ISI (right) for the three groups of AON neurones.

569

570 **Figure 3. Type 2 neurones.**

571 **A,** The rhythmic discharge cell of a typical type 2 neurone. The cell fired in bursts of 2-
572 20 spikes with a period of 1.5 s. **B,** ISI distribution of the neurone in A. **C,** Consensus ISI
573 distribution for 45 type 2 cells (means ± SE). **D**, Autocorrelation histogram for the
574 neurone in A,B. **E,** Average normalised autocorrelations for 45 type 2 cells (means ± SE).
575 **F,** Pair of type 2 neurones, both displaying bursts with a period of about 1 s. Bursts in
576 the smaller cell begin about 200 ms before bursts in the larger cell. **G,** Cross-
577 correlograms (in 10-ms bins) showing activity in each of the neurones in F relative to
578 spikes in the other, constructed over 300 s of spontaneous activity. Both neurones
579 discharged in bursts in a rhythm with a period of about 1 s, but out of phase by about 200
580 ms. Bursts in cell 1 (smaller spikes in F) preceded bursts in cell 2 (larger spikes in F). The

dotted line shows timing of spikes in cell 1 relative to spikes in cell 2 and the solid line shows spikes in cell 2 relative to cell A.

Figure 4. Responses of AON cells to bedding odour.

A, Example of the response of a type 1 cell to bedding odour (grey bar). **B,** Averaged responses (± SE) of ten type 1 cells that were excited by bedding odour. **C,** example of an inhibitory response (one of two cells inhibited). **D,** Response of a type 2 cell that was excited after the end of odour application. **E,** Response of the same cell to LOT stimulation – the cell was inhibited during stimulation but showed excitation after stimulation.

Figure 5. Effects of LOT stimulation on type 1 neurones.

A, Extract of voltage trace showing spike responses (blue arrows) during a train of stimuli (red arrows) at 50 Hz. **B,** Post-stimulus time histogram of the response of a type 1 neurone activated by stimulation of the LOT with a latency of about 7 ms. **C,** A neurone inhibited by LOT stimulation (red bar) at 50 Hz. **D,** A neurone activated after the end of LOT stimulation (red bar) **E,** A neurone excited during LOT stimulation (red bar) but inhibited after. **F,** Expanded voltage trace from E showing that each stimulus pulse (red arrows) is followed by a spike (the first indicated by the blue arrow). **G,** A neurone progressively excited during LOT stimulation (red bar) with prolonged post-stimulus activation. The top trace shows detected spikes. **H,** This neurone was synaptically activated by LOT stimulation (red arrows) but each activation was followed by inhibition. **I,** Expanded voltage trace from H showing spike (blue arrow) following stimulus (red arrow).

Figure 6. Response of a type 1 cell to odours and stimulation of the LOT.

A, This neurone was repeatably activated by bedding odour (blue bars), unaffected by air (yellow bar) or valderaldehyde (val, orange bar) and weakly activated by garlic (green bar). **B,** Expansion of the response to bedding showing clear rhythmic discharge during odour application. **C,** Response to trains of stimulation at the frequencies shown. During tests, the cell fell silent and was unresponsive to brief stimulation at 20, 30 and 50 Hz but was activated after stimulation at 150 and 200 Hz. **D,** Responses to continued lower frequency stimulation. Stimulation at 10 and 20 Hz produced excitation after a delay. **E,** Voltage record of the response during activation by 20 Hz stimulation – the stimulus artefacts are marked by the red arrowheads. **F,** Peri-stimulus histograms of responses to stimulation at 10 Hz (green) and 20 Hz (blue, expressed as the probability of a spike occurring in 1-ms bins (P_{spike}). The stimulus time is marked by the red bar.

Figure 7. Response of a type 2 cell to stimulation of the LOT.

A, Extract of spontaneous activity of a type 2 neurone showing intermittent bursts with a period of about 2 s. **B,** Autocorrelation histogram, typical of type 2 cells. **C,** ISI distribution, shows the typical unimodal, skewed distribution. **D,** During 1 Hz stimulation of the LOT (artefacts marked in red in voltage trace) the cell initially was unresponsive. **E,** With continued stimulation, bursts arose entrained by the stimuli. **F,** Peri-stimulus time histogram, revealing post stimulus inhibition followed by prolonged excitation. **G,** The response to a brief train of stimulation at 50 Hz, showing delayed activation that outlasted the simulation.

Figure 8. Type 1 neurones that are activated during inspiration are activated by

LOT stimulation.

A, Extract of recording of respiratory activity in conjunction with recording the spontaneous activity of a type 1 neurone. **B,** Identification of this neurone as activated by LOT stimulation. The shaded bar covers the stimulus artefact accompanying a stimulus pulse applied to the LOT; the pulse is followed by two spikes from this neurone (arrowed) and a smaller spike (with a negative going waveform) from a second, almost silent neurone. **C,** ISI distribution from this neurone, typical of type 1 neurones. **D,** Spike activity relative to respiratory cycle. During 300 s of spontaneous activity, the time of the peak inspiration in each respiratory cycle was marked, and the dotted line shows the average cycle produced from these triggers. The histogram shows the spike activity in 10-ms bins triggered in the same way. **E,** Respiration relative to spike activity. The solid line shows the conventional autocorrelation histogram for this cell – this is a spike-triggered average of spiking activity. The dotted line shows the directly corresponding spike-triggered average of respiratory activity. Both D and E show, in slightly different ways, that the bursts in this cell were consistently initiated during inspiration.

Figure 9. Type 1 neurones that are activated during expiration are inhibited by LOT stimulation.

A, Extract of recording of respiratory activity in conjunction with recording the spontaneous activity of a type 1 neurone. **B,** Identification of this neurone as inhibited by LOT stimulation. Peristimulus time histogram of spike activity (in 10-ms bins) of 300 s of activity during 1 Hz LOT stimulation. The shaded bar covers the stimulus artefact accompanying stimulation; the pulse is followed by about 50 ms of inhibition. **C,** ISI distribution from this neurone, typical of type 1 neurones. **D,** Spike activity relative to

653 respiratory cycle. During 300 s of spontaneous activity, the time of the peak inspiration
654 in each respiratory cycle was marked, and the dotted line shows the average cycle
655 produced from these triggers (increased signal corresponds to inspiration). The histogram
656 shows the spike activity in 10-ms bins triggered in the same way. **E,** Respiration relative
657 to spike activity. The solid line shows the conventional autocorrelation histogram for this
658 cell – this is a spike-triggered average of spiking activity. The dotted line shows the
659 directly corresponding spike-triggered average of respiratory activity. Both D and E show,
660 in slightly different ways, that the bursts in this cell were consistently initiated during
661 expiration.
662
663 **Figure 10. The importance of phase-shifts.**
664 The mitral cells of the MOB fire spontaneously in a rhythm locked to the inspiration phase
665 of the respiratory cycle, and each is narrowly tuned to a specific odour. In the AON,
666 neurones receive convergent inputs from multiple mitral cells, making them more broadly
667 tuned. However, the synchronicity of spontaneous activity rhythms presents a problem
668 illustrated in **A**. The black traces simulate the activity of eleven 'cells' all firing at the
669 same phase of the rhythm. One of these cells (the bottom trace) is activated by an odour
670 in the period marked by the yellow bar. But the aggregate activity (in blue), as seen by a
671 second order neurone, the signal is virtually invisible. The signal is diluted by a factor of
672 1:11. In **B,** the same activity of the eleven 'cells' is shown, but each cell is phase shifted
673 (red line). Now the signal is clearly apparent in the aggregate activity. The signal is still
674 diluted, but by a much smaller factor of about 1:3. For simple simulation, each 'cell' was
675 generated by assuming that the probability of 'spiking' was determined by a sinusoidal
676 input.

677